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ECO-FRIENDLY BIOPROCESSING OIL PALM EMPTY FRUIT BUNCH (OPEFB) FIBERS INTO NANOCRYSTALLINE CELLULOSE (NCC) USING WHITE-ROT FUNGI (*TREMETES VERSICOLOR*) AND CELLULASE ENZYME (*TRICHODERMA REESEI*)

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Abstract. *The oil palm empty fruit bunch (OPEFB) as solid biomass of palm oil mill industry is available in abundance and has the potential to be utilized as the raw material of nanocrystalline cellulose (NCC). This research aims to investigate the effect of bioprocess treatment (bio-delignification, bio-bleaching, and enzymatic hydrolysis) on the nanocrystalline cellulose synthesized from OPEFB. The bio-delignification of OPEFB fiber was carried out using white-rot fungi (Tremetes versicolor and pre-bleaching pulp with xylanase. Trichoderma reesei, a cellulase enzyme type was used to hydrolyze the OPEFB fiber into nano-sized cellulose. The result exhibits that the cellulose content of OPEFB pulp using bio-delignification increased significantly compared to chemical treatment. Furthermore, the concentration of enzyme and hydrolysis time in the synthesis treatment affect reducing average particle size and increasing the crystallinity index while decreasing the yield of NCC produced. The synthesis process was under optimal processing conditions at 1% enzyme concentration and 3 days of hydrolysis time resulting in the NCC product with 155 nm of average particle size, 66.78% of crystallinity index, and a yield of 38.28%. The bioprocess technology applied in this study could improve the cellulose yield of OPEFB and enhance the quality parameters of NCC products such as particle size and crystallinity index.*

Keywords: *bio-delignification; hydrolysis; white-rot fungi; nanocellulose crystalline; OPEFB*

1. Introduction

Indonesia is a major palm oil producer, which contributes to the abundant solid waste total of oil palm empty fruit bunch (OPEFB), and accounts for approximately 22-35% of hemicellulose content [1]. Cellulose and its derivatives can be considered and investigated as an inexhaustible material due to its superiority in its renewability, biodegradability, and abundance availability [2].

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Cellulose-based materials have been used in a wide range of applications, including composites, packaging, aerogels, hydrogels, fibers, tissue engineering, membranes, textiles, and coatings [3].

There are several factors influencing the use of OPEFB, i.e., intrinsic factors, including high cellulose content and potential availability, and extrinsic factors, including environmental and energy concerns, which drive the development of environmental-friendly technology. Due to its high cellulose content, recyclability, and environmental friendliness, OPEFB is regarded as a valuable organic waste (bio-renewable resources) and considered a raw material to be converted into other cellulose-based raw materials, such as nanocrystalline cellulose (NCC) [4]. Even though the OPEFB cellulose is not plastic, structural modifications are required to make it so and cellulose fiber modification technology was developed to reduce the cellulose fiber particle size [5].

Nanocrystalline cellulose (NCC) is a nano-sized cellulose biomaterial with satisfactory mechanical properties essentially high tensile strength, modulus, and biodegradability, and can be utilized in a variety of medical and pharmaceutical products, packaging, composites, paper, and food as a reinforcing material, filler, and carrier. It is also used as a base material in biopolymer nanocomposites due to its nano dimensions, excellent mechanical properties, highly reactive surface, and biodegradability [6]. Numerous types of studies focusing on the isolation, processing, and characterization of nanocrystalline cellulose have been conducted concerning the advancement properties of NCC, including mechanical, chemical, biological, and hybrid methods [7]. The cellulose synthesis methods have significant impacts on increasing production yield, as well as their remarkable properties such as high surface chemistry, physical properties, biocompatibility, and biodegradability [8], [9]. There are many factors influencing the nanocellulose properties, including the fiber type, environmental conditions, synthesis methods, and adjustment of the fiber surface [10]. Recent research has found that biodegradable nanocellulose products outperform nonbiodegradable composite materials in terms of physical, mechanical, and thermal properties [11].

Synthesizing nanocellulose from lignocellulose has been identified as a potential issue for sustaining the economy and society. Nonetheless, using chemical methods has significant drawbacks, including the use of toxic chemicals that are harmful to the environment, high costs and energy consumption, and generation of wastewater [12]. To address these issues, more research is required and has been conducted and developed, such as the sonication-hydrothermal [13], [14], radiation-enzymatic [15], and enzymatic methods [16]. These methods entail chemical processes, particularly in the pulping of raw materials to produce cellulose. In the future, the environmental-friendly engineering process technology in nanocrystalline cellulose production will be both a challenge and an opportunity.

The biological pretreatment of lignocellulose from Oil Palm Empty Fruit Bunch (OPEFB) fiber is required and considered effective because it is slight, substrate-specific, and does not produce any inhibitory chemicals that would disrupt enzymatic activities or the fermentation process [17]. Bio-pulping is the fungal pretreatment that limits the number of white-rot fungi that colonize and degrade lignin in wood while preserving cellulose and is considered an environmental-friendly technology to overcome issues associated with conventional chemical and mechanical pulping methods [18]. Meanwhile, bio-bleaching employs hemicellulolytic enzymes, particularly xylanase, which is commercially available and has been demonstrated to be effective in the bleaching [19]. White rot fungi are commonly used in this process because they can degrade all wood components including lignin, cellulose, and hemicellulose simultaneously by producing enzymes [20], [21]. The following are the benefits of the bio-pulping process using white-rot fungi: it can be used for hardwoods and softwoods, reduce refining time, increase 30% of energy savings in mechanical pulping, increase fiber physical properties, reduce the chemicals uses in chemical pulping, and reduce pitch content [22], [23].

Since it is simple and effective in reducing the use of bleaching chemicals, bio-bleaching enzyme technology was introduced for the bleaching process. Hemicellulase (xylanase), ligninolytic (laccase), lipase, and α-glucuronidase are some of the enzymes used in the bio-bleaching process. The use of xylanase on non-wood mash is also known to reduce the number of synthetic chlorine compounds used. Because of a few specialized, financial, and natural advantages in the paper industry, the use of xylanase for the bio-bleaching process is rapidly advancing. From an ecological standpoint, the use of xylanase can reduce the amount of Adsorbable Organic Halides (AOX) present [24], when compared to controls by 21.4-26.6% [25], Chemical Oxygen Demand (COD), Biological Oxygen Demand (BOD), and increasing the proportion of BOD and COD in abundant when compared to control, demonstrating that the gushing becomes more straightforward to be corrupted naturally in optional treatment [26].

There are many distinctive investigations that have been conducted to mitigate the adverse effects and drawbacks of the preceding methods. Nanocellulose can be produced using the enzymatic method by precisely cutting the amorphous portion of a cellulose chain. An eco-friendly method of cellulase enzyme hydrolysis has been investigated to produce nanocrystalline cellulose from lignocellulose materials by controlling pH, temperature, and time conditions to replace the chemical hydrolysis process [16]. The cellulase enzyme contributes to the catalysis of the breakdown of the cellulose [27].

Based on the preceding studies, there is an opportunity to conduct a prominent and more eco-friendly method by developing the bioprocess method of nanocrystalline cellulose from OPEFB. The goal of this study is to investigate the effects of using bioprocess technology on the nanocrystalline cellulose produced from oil palm empty fruit bunch (OPEFB) solid biomass which is maintained from the local palm oil mill in West Sumatera, Indonesia. The biological treatment involved in this research includes bio-pulping using white-rot fungi (Tremetes versicolor), bio-bleaching using xylanase enzyme, and enzymatic hydrolysis using cellulase enzyme type, Trichoderma reesei. The intermediate product from bio-pulping treatment will be compared to the standard using the chemical treatment, and the final products of NCC will be characterized by their physical properties, such as morphological structure, particle size, crystallinity index, and yield.

2. Methods

2.1 Materials and equipment

This experiment employed oil palm empty fruit bunch (OPEFB) as raw material derived from the local palm oil industry in West Sumatera, Indonesia. The other materials employed in this experiment were the white-rot fungi (Tremetes versicolor), xylanase enzymes (Sigma), cellulase enzymes (Trichoderma reesei) (Sigma), potato dextrose agar (Himedia), NaOH, ClO_2 and distilled water. The types of equipment used were hotplate stirrers, glassware, thermometer, pH meter, autoclave, sonicator bath, mesh filter, grinding mill, and analytical balance.

2.2 Methods

This research was conducted in three steps include the preparation of OPEFB fibers, the bio-pulping and bio-bleaching of OPEFB fibers, and enzymatic hydrolysis. The following described the procedure used in this study.

2.2.1 The preparation of OPEFB fibers

The OPEFB fiber was washed to remove dirt and dried for two days until entirely dry. The size of OPEFB fibers was then reduced using a grinding mill. The chopped OPEFB fiber was then sieved through 20 and 45 mesh sieves to attain a homogeneous size range of 0.85 mm to 0.35 mm. The fiber was then dried in an oven at 105°C for 2 hours, or until the sample weight remained constant.

2.2.2 The bio-pulping and bio-bleaching of OPEFB fibers

The white rot-fungi *T. versicolor* was used in the bio-pulping process. 150 g sample was placed in a heat-resistant glass container, immersed in distilled water for 24 hours, and sterilized in an autoclave for 15 minutes at 121°C. Furthermore, it was inoculated with 100 ml *T. versicolor* suspension in a PDA medium that formerly had grown for 7 days in an Erlenmeyer flask. The samples were then incubated for 20 days at room temperature. The content of lignin, hemicellulose, and cellulose in samples before and after delignification was determined. Fungi-free controls were treated under the same condition and analyzed on day 20 [28]. The cellulose standard used for comparison was cellulose from the chemical pulping process using 14% NaOH for 60 minutes of residence time. Subsequently, the bio-bleaching process was carried out using 180 grams of pulp using 1 kg/ton of pulp xylanase [29] and bleached with an Elemental Chlorine Free (ECF) system. The conditions were kept constant: 10% consistency, 50°C temperature, and a reaction time of 60 minutes. Henceforth, the pulp was bleached using the bleaching stage as shown in Table 1.

Table 1. Bleaching process D_0 E D_1 D_2 [30]

Bleaching process	Chemicals	Dose (%)	Time (minute)	Temperature (°C)	Consistency (%)
D_0 (initial chlorine dioxide)	ClO_2	0,22 Kappa Number/2,63	60	60	10
E (extraction)	NaOH	1	60	70	10
D_1 (chlorine dioxide 1)	ClO_2	1	180	75	10
D_2 (chlorine dioxide 2)	ClO_2	0,5	180	75	10

2.2.3 Enzymatic hydrolysis

The enzymatic hydrolysis process of the Trichoderma reesei enzyme was carried out at this stage [16], [31]. The steps employed in the production of nanocellulose are the following: cryo-crushing, enzyme addition, and sonication for 30 minutes. As the enzyme was introduced, OPEFB fiber was suspended in Na-citrate buffer and treated with 0.5% and 1% crude extracts of the cellulase enzyme from Trichoderma reesei. Afterward, OPEFB fiber was incubated for 2, 3, and 4 days at 50°C and 150 rpm for agitation speed. Tremetes versicolor white-rot fungi were used in the bio-pulping process. Particle Size Analyzer (PSA), Scanning Electron Microscopy (SEM), crystallinity (X-Ray Diffraction), and yield were all used to characterize the NCC.

2.3 Nanocellulose Crystalline (NCC) Characterization

2.3.1 Particle Size Analyzer (PSA)

It was used to quantify the particle’s infinite size conveyance using Vasco's Particle Size analyzer brand with Serial Number: PSA114102.

2.3.2 Scanning Electron Microscopy (SEM)

This characterization was used to measure and visualize the dimensions of the NCC using a Zeiss SEM model EVO MA 10 with a magnification of 50,000x.

2.3.3 X-Ray Diffraction (XRD) Characterization

This characterization was used to determine the particle size and identify the crystalline phase in the material by determining the lattice structure parameters. The Xpert Pro Analytical PW30 / 40 X-RD brand was used for this test. The equation used for calculating the sample's crystallinity index is as follows [32].

$$CrI = \frac{I_{002-}I_{AM}}{I_{002}} x100$$

where:

CrI = relative degree of crystallinity

I_{002} = maximum intensity of diffraction pattern 0 0 2

I_{AM} = intensity of diffraction in similar unit at 12-18°

3. Results and Discussion

3.1 OPEFB Cellulose

Oil palm empty fruit bunches (OPEFB) are solid biomass derived from oil palm processing plants. It accounts for 25-26% of processed fresh fruit bunches. The results exhibit that the cellulose content of OPEFB was 38.7%. Due to its high cellulose content, it has the potential to become raw material for cellulose extraction. Cellulose is an important raw material used in a variety of industrial sectors, including the nanocrystalline cellulose [33].

Figure 1. Production of OPEFB fiber

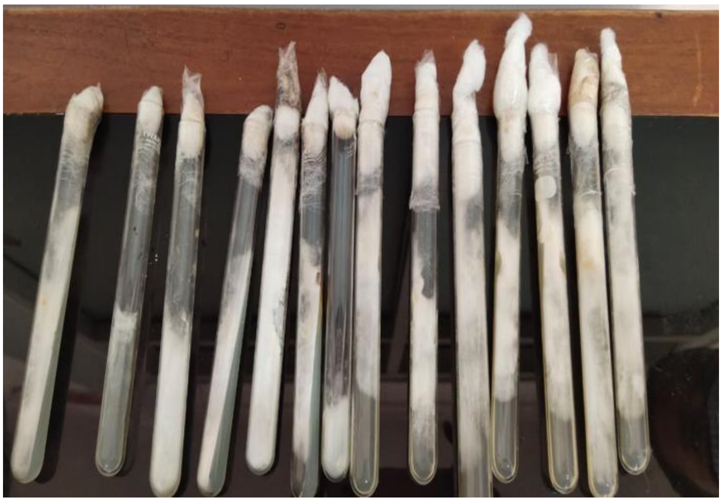

Figure 2. Inoculation results using *T. versicolor* fungi

The first stage of this research was processing oil palm empty fruit bunches into fibers to smaller sizes using a grinding mill, resulting in fiber sizes ranging from 0.35 to 0.85 mm, as presented in Figure 1. Cellulose from OPEFB can be purified (pulped) in two stages: delignification followed by bleaching. In this study, bio-delignification was applied to dissolve entire components of OPEFB other than cellulose. This process employed white rot fungi from Tremetes versicolor to bio-delignify OPEFB. Figure 2 depicts the results of the fungi inoculation after 7 days. This fungus has white hyphae based on visual observations of its growth.

The OPEFB fiber samples were then incubated for 20 days at room temperature with the white-rot fungi T. versicolor, with the results presented in Figure 3. Tremetes versicolor, a white-rot fungus, began to grow and spread across the OPEFB fibers. The fiber lignin content was strongly reduced after 20 days of incubation. This can be seen in the fibers attacked by the fungus, which exhibited a yellowish-white color when compared to the initial fibers. White rot fungi's enzyme attack power can be increased by shrinking the fiber. This was consistent with the observations of Foody et al [34], who stated that physical treatment, essentially size reduction, contributes to lowering lignin content and shortening the delignification process time. Rot fungus is a biological agent that can be used to delignify OPEFB’s fibers as an alternative. Laccase enzymes produced by rot fungi can reduce solid waste from palm oil processing by producing more valuable products.

Figure 3. Fiber appearance after 20 days of incubation

Bio-pulping is a pulping method that uses biological agents to produce cellulose and hemicellulose. The main key in the bio-pulping process is bio-delignification using biological agents. To obtain pure cellulose, OPEFB fiber that had been bio-delignified with Tremetes versicolor was subjected to a chemical process that dissolved lignin using the soda method for 30 minutes and NaOH concentration of 14%. Many studies have further exposed that using biological agents in the pretreatment of lignocellulosic biomass prior to mechanical or chemical processes can reduce energy consumption, pollution, and increase paper strength [35], [36].

Bleaching process using the xylanase enzyme was used to remove pigment and lignin residue. The methods used were pre-bleaching pulp with xylanase and pulp bleaching with an ECF (Elemental Chlorine Free) system. Chlorine dioxide, the main chemical in ECF bleaching technology, has excellent selectivity in delignification. Pulp bleaching with xylanase is a typical method to produce bleached pulp. The brightness of the pulp increased after the bleaching process. It indicated that the pigment and lignin had dissolved, and thus high purity cellulose was obtained.

Xylanase is a hemicellulose enzyme that hydrolyzes xylan, which is then re-deposited on the surface of pulp microfibrils. Bleaching with xylanase resulted in the breakdown of hemicellulose chains between lignin and complex carbohydrates, removing lignin and reducing bleaching chemical consumption. By incorporating enzymes into pulp, it is possible to save 15% of active chlorine in one stage of bleaching and 18.7% in multiple stages while achieving the same brightness as the control pulp [37]. Several research indicated that xylanase has a high potential in the pulp industry's bleaching process. The presence of xylan inhibits cellulose enzymatic hydrolysis, as exhibited by an increase in cellulose crystallinity and preserved crystal width measured with wide-angle X-ray scattering. Purified xylanase was predicted to remove a loosely bound fraction of xylan, resulting in proportional hydrolysis of xylan and cellulose with the cellulase mixture [38].

Table 2. OPEFB pulp composition

OPEFB pulp composition	Biopulping (%)	Standard (%)
Lignin	11.37	12.28
Cellulose	68.23	62.14
Hemicellulose	20.26	25.12

Table 2 presents the composition analysis of OPEFB pulp after bio-pulping in comparison to the standard process, including lignin, hemicellulose, and cellulose. The cellulose content was 88.49%, consisting of cellulose and hemicellulose, which was slightly higher than the standard obtained through chemical processes. The yield obtained in this study was comparatively high, reaching 90% of the total cellulose content of OPEFB due to the usage of chemical agents that can dissolve cellulose during the pulping and bleaching processes. It outperformed the standard yield, which only seized at 80%, and improved pulp quality.

3.2 Nanocrystalline Cellulose (NCC)

Enzymatic hydrolysis of *Trichoderma reesei* enzyme was used to synthesize nanocrystalline cellulose. The product of NCC was synthesized in two different forms, i.e., solution and powder, as presented in Figure 4. A spray dryer was used to dry the powder NCC. Sonication was used to disperse the production of cellulose nanocrystals after cellulose hydrolysis. The effect of sonication on particle size, which tends to be more homogeneous and shrinking, resulted in more stable nanoparticle size and less agglomeration. Sonication waves are capable to disintegrate agglomerations and generate complete dispersion. An ultrasonic bath with a high frequency of 20 kHz was used in this method. It employed wave energy for the cavitation process, which aimed to form small bubbles due to ultrasonic wave transmission.

Figure 4. The NCC product, (1) solution form; (2) powder form

Table 3. Result of particle size, crystallinity, and yield of NC

Treatment	Average of particle size (nm)	Crystallinity index (%)	Yield (%)
enzyme concentration : 0.5%			
2 days	371.63	53.64	42.15
3 days	224.40	60.15	40.84
4 days	166.77	66.87	36.37
enzyme concentration: 1%			
2 days	309.72	60.12	41.33
3 days	155.00	66.78	38.28
4 days	79.19	68.22	34.56

Table 3 exhibits the effect of various enzyme concentrations and hydrolysis time on the particle size, crystallinity index, and yield of NCC. Higher enzyme concentration reduces particle size and yield while increasing the crystallinity index. The dispersion of NCC size was determined by using Particle Size Analyzer (PSA) and the results are illustrated in Figure 5.

The crystallinity index (CI) value calculated from the XRD results using the Segal method is presented in Figure 6. The CI value is an important physical parameter to be measured due to its correlation to the thermal and mechanical properties [39]. By considering the process's efficiency and high enzyme value, the process can be optimized with an enzyme concentration of 1% and 3 days of residence time while other parameters were kept constant, such as the temperature of 50°C, pH of 5, and agitation speed of 150 rpm. The NCC obtained had an average size of 155.0 nm with an improved crystallinity index of 66.78%.

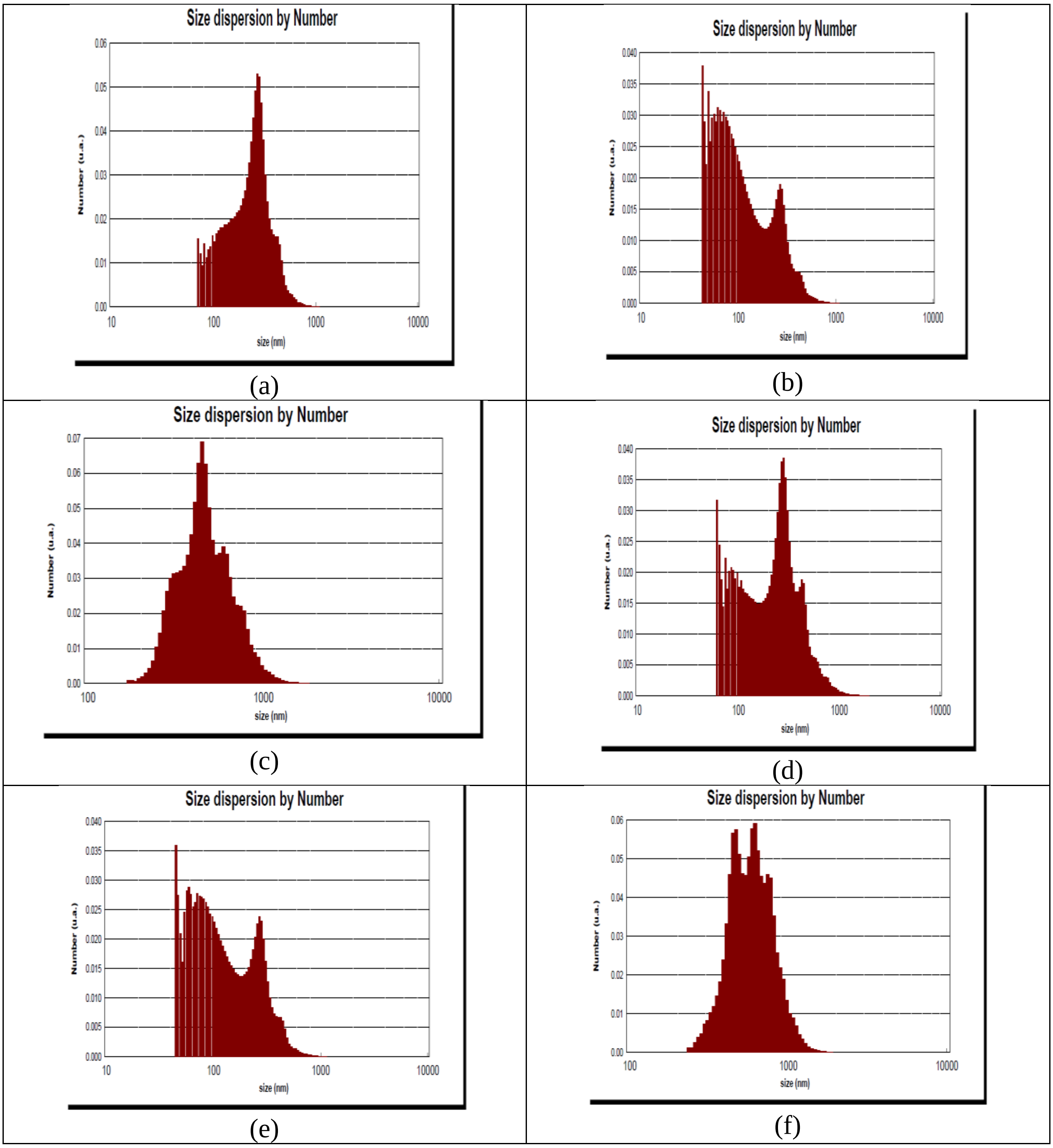

Figure 5. The Particle Size Analysis (PSA) results of NCC with various enzyme concentration and hydrolysis time, (a) 0.5%, 2 days; (b) 0.5%, 3 days; (c) 0.5%, 4 days; (d) 1%, 2 days; (e) 1%, 3 days; and (f) 1%, 4 days.

To replace the chemical hydrolysis process, a more environmental-friendly cellulase enzyme hydrolysis method was used to convert cellulose into nanocrystalline cellulose by controlling pH, temperature, and time conditions. The amorphous form of cellulose was broken down using a group of enzymes known as -1,4 glucan-4-glucan hydrolase or cellulase enzymes. Enzymatic hydrolysis of bacterial cellulose with *Trichoderma reesei* can yield nano cellulose with dimensions of 100-300 nm x 10-15 nm [16]. To reduce the size of microcrystalline cellulose (MCC), the enzymatic method with the *Trichoderma reesei* enzyme was used. The nanocellulose particle size were obtained at 313.0 49.6 nm and 1209 155.8 nm. Cellulase enzymes were used to breakdown the cellulose [31]. The addition of cellulase enzyme concentration to the hydrolysis process can increase the rate of hydrolysis while decreasing yield and escalating costs. The morphology structure of each sample NCC is characterized using Scanning Electron Microscopy (SEM) at 50,000 magnification and the results are depicted in Figure 7.

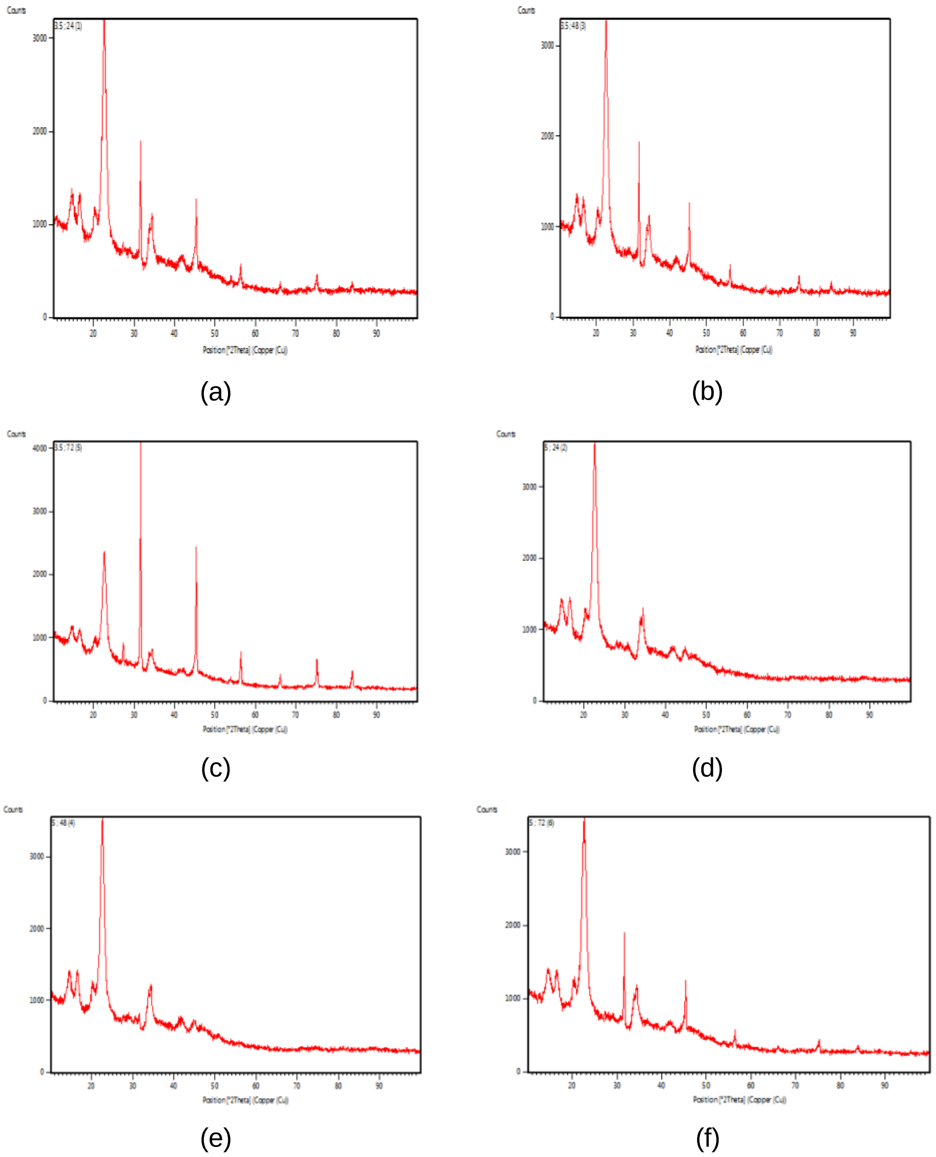

Figure 6. The X-Ray Diffraction (XRD) results of NCC with various enzyme concentration and hydrolysis time, (a) 0.5%, 2 days; (b) 0.5%, 3 days; (c) 0.5%, 4 days; (d) 1%, 2 days; (e) 1%, 3 days; and (f) 1%, 4 days

Enzymatic hydrolysis is preferred over acid hydrolysis because the enzyme exclusively works on a specific product, resulting in no unexpected products, and it can also be used in lower processing conditions. The cellulase enzyme is used in the enzymatic hydrolysis process to break down cellulose into its monomers. The use of enzymes for hydrolysis is accomplished by simply replacing the acid hydrolysis stage with the enzyme hydrolysis stage. Enzymatic hydrolysis has several advantages over acid hydrolysis, including no sugar degradation from hydrolysis, lower processing conditions (pH of 5 and temperature of 45-50°C), no side reactions, and the use of noncorrosive materials. Some disadvantages of enzymatic hydrolysis include the fact that the process takes longer, and that the product inhibits the enzyme's work. Furthermore, enzymes work specifically and cannot penetrate lignin-bound cellulose and hemicellulose. Delignification is the process of removing lignin from lignocellulose prior to the enzymatic hydrolysis process. The cost of enzymes, which is higher than that of acid, is another disadvantage of using enzymatic hydrolysis. Cellulose can be hydrolyzed enzymatically using cellulase enzymes. Microbes that produce cellulase enzymes, such as *Trichoderma reesei*, *Trichoderma viride*, and *Aspergillus niger*, can be used to hydrolyze cellulose [40], [41]. Temperature, pH, enzyme concentration, and reaction time are all causative factors in enzymatic hydrolysis.

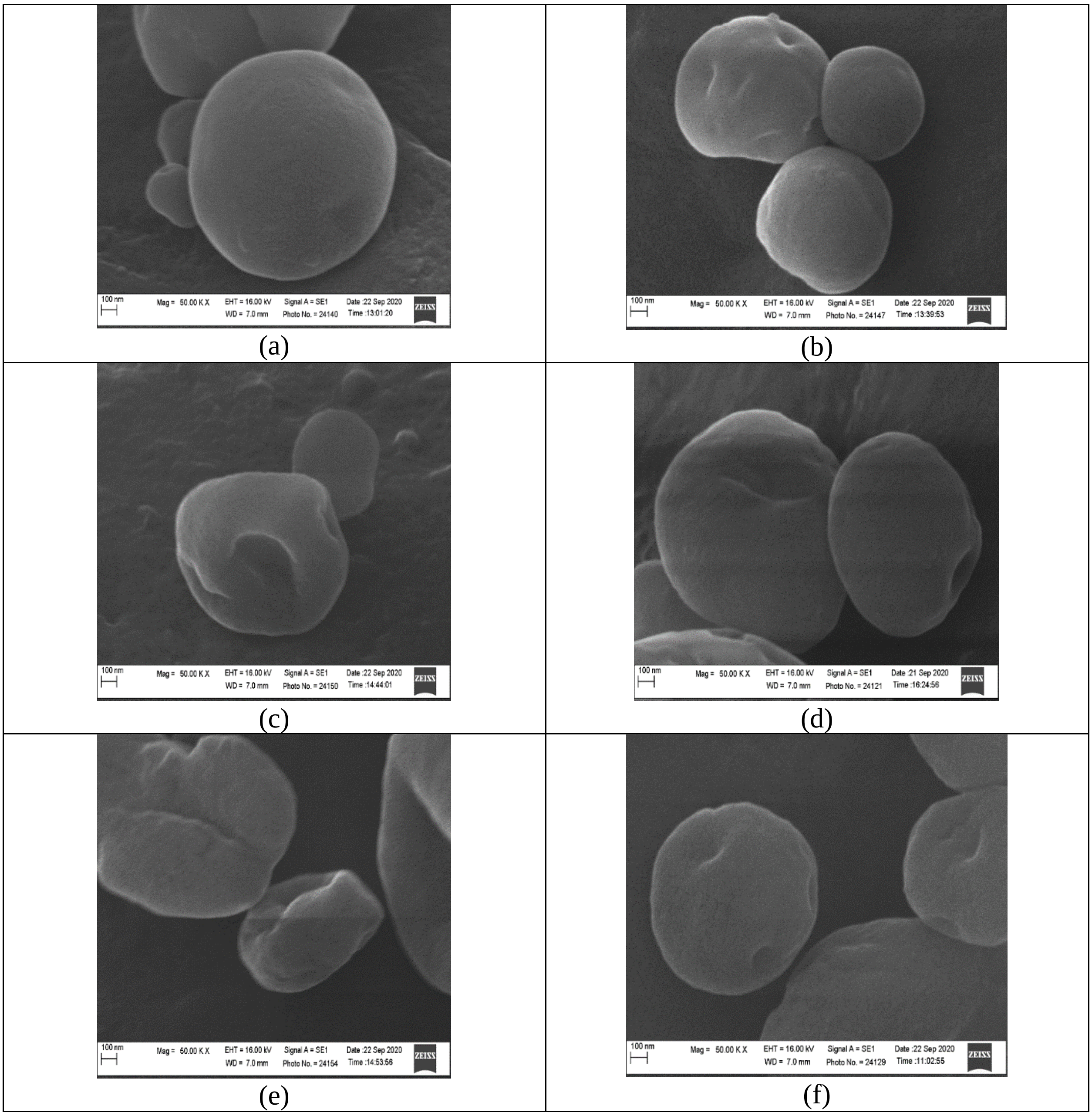

Figure 7. The SEM results of NCC with various enzyme concentration and hydrolysis time, (a) 0.5%, 2 days; (b) 0.5%, 3 days; (c) 0.5%, 4 days; (d) 1%, 2 days; (e) 1%, 3 days; and (f) 1%, 4 days.

4. Conclusions

The production of nanocellulose crystalline (NCC) from solid biomass of OPEFB using bioprocess treatment has been carried out successfully. The bioprocess technology applied in this research includes bio-delignification with *Tremetes versicolor* (white-rot fungi), bio-bleaching with pulp pre-bleaching with xylanase, and hydrolysis with *Trichoderma reesei* (cellulase enzyme type). The findings of this research shows that using Tremetes versicolor as the biological agent in the delignification of OPEFB fiber can degrade lignin and produce more cellulose content compared to the chemical treatment, and the product of NCC can be obtained under optimal conditions of 0.5% enzyme concentration and 3-days of residence time, resulting 155 nm of average particle size, 66.78% of crystallinity index, and the yield of 38.28%. The bioprocess technology applied in this study has the potential to increase the cellulose yield of OPEFB while also improving the quality parameters of NCC products such as particle size and crystallinity index.

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